PB87-145322

Applications for DNA Probes in Biodegradation Research

Tennessee Univ., Knoxville

Prepared for

Environmental Research Lab., Gulf Breeze, FL

Dec 86

U.S. Department of Commerce
National Technical Information Service
NTIS

EPA/600/D-86/249
December 1986

PB87-145322

Applications for DNA Probes in Biodegradation Research

Gary S. Sayler*, Rakesh K. Jain, Andrew Ogram,

Charles A. Pettigrew, Laura Houston,

James Blackburn, and William S. Riggsby

The Department of Microbiology and

The Graduate Program in Ecology

The University of Tennessee

Knoxville, TN 37996 USA

(615) 974-5219

*Corresponding author

Environmental Research Laboratory
Office of Research and Development
U.S. Environmental Protection Agency
Gulf Breeze, FL 32516

REPRODUCED BY
U.S. DEPARTMENT OF COMMERCE
NATIONAL TECHNICAL
INFORMATION SERVICE
SPRINGFIELD, VA. 22161

TECHNICAL REPORT DATA
(Please read Instructions on the reverse before completing)

1. REPORT NO. EPA/600/D-86/249	2.
3. RECIPIENT'S ACCESSION NO. PB87 145322/AS	
4. TITLE AND SUBTITLE APPLICATIONS FOR DNA PROBES IN BIODEGRADATION RESEARCH	5. REPORT DATE December 1986
	6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S) G.S. Sayler, R.K. Jain, A. Ogram, C.A. Pettigrew, L. Houston, J. Blackburn, and W.S. Riggsby	8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS Dept. of Micro. & Graduate Prog. in Ecology, Univ. of Tenn., Knoxville, TN 37996	10. PROGRAM ELEMENT NO.
	11. CONTRACT/GRANT NO. CR 812488
12. SPONSORING AGENCY NAME AND ADDRESS Environmental Research Laboratory Office of Research and Development U.S. Environmental Protection Agency Gulf Breeze, FL 32516	13. TYPE OF REPORT AND PERIOD COVERED
	14. SPONSORING AGENCY CODE EPA-600/04

15. SUPPLEMENTARY NOTES

16. ABSTRACT

The use of DNA:DNA hybridization technology in biodegradation studies is investigated. The rate constants for sediments exposed to synthetic oils could be calculated from the NAH^+ genotypes and this approach would be useful in predicting the kinetics of aromatic hydrocarbon degradation. Gene probes prepared from NAH7 plasmid were also used to monitor and enumerate the naphthalene-degrading populations in a continuous mixed culture bioreactor and this analysis demonstrated at least one order of magnitude difference in the naphthalene-degrading population over the conventional plate analysis. It was also shown that using pSS50 (a chlorobiphenyl mineralizing plasmid) as probe DNA, other polychlorinated biphenyls degrading organisms can be identified from the environment. Further, the maintenance and stability of a genetically modified Pseudomonas putida (carrying plasmids TOL and RK2) over an 8-week period in chemically contaminated groundwater aquifer material was established. Results demonstrate the wide applications, significance, sensitivity, and accuracy of DNA proves in environmental biodegradation research.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group

18. DISTRIBUTION STATEMENT RELEASE TO PUBLIC	19. SECURITY CLASS *(This Report)* UNCLASSIFIED	21. NO. OF PAGES 35
	20. SECURITY CLASS *(This page)* UNCLASSIFIED	22. PRICE

EPA Form 2220-1 (Rev. 4-77) PREVIOUS EDITION IS OBSOLETE

ABSTRACT

The use of DNA:DNA hybridization technology in biodegradation studies is investigated in the present work. The rate constants for sediments exposed to synthetic oils could be calculated from the NAH^+ genotypes and this approach would be useful in predicting the kinetics of aromatic hydrocarbon degradation. Gene probes prepared from NAH7 plasmid were also used to monitor and enumerate the naphthalene-degrading populations in a continuous mixed culture bioreactor and this analysis demonstrated at least one order of magnitude difference in the naphthalene-degrading population over the conventional plate analysis. It was also shown that using pSS50 (a chlorobiphenyl mineralizing plasmid) as probe DNA, other polychlorinated biphenyls degrading organisms can be identified from the environment. Further, the maintenance and stability of a genetically modified Pseudomonas putida (carrying plasmids TOL and RK2) over an 8-week period in chemically contaminated groundwater aquifer material was established; this involved the use of specific DNA probes for TOL and RK2. Finally, a novel approach to detect and quantitatively determine the DNA sequence homologous to specific probe DNA in the total genomic DNA extracted from a complex microbial community has also been developed. Results obtained here demonstrate the wide applications, significance, sensitivity, and accuracy of DNA probes in environmental biodegradation research.

INTRODUCTION

Research goals in biodegradation are multifaceted and may be focused on basic or applied objectives. Such goals may include:

(1) Predicting fate and exposure concentrations of chemical contaminants relative to environmental toxicology.

(2) Developing treatment processes for destruction or transformation of pollutants.

(3) Predicting microbial community and ecosystem alterations or response to environmental pollution.

(4) Measuring the rate of adaptation or evolutionary change of microbial populations as selected by ecological and environmental factors.

These multifaceted goals require a quantitative basis for successful prediction or extrapolation in the larger contents of ecology and environmental science. Yet, in many instances the outcome of research in the area of biodegradation is qualitative or at best system specific at a quantitative level. The result is that the field has generated a great deal of data to support arguments that a given organic compound can or cannot be degraded in this or that environment; and if quantitative data are available, the rates of degradation may range across several orders of magnitude. Neither outcome leaves investigators in the field or the technical audience with much confidence in real world predictions.

There is a need to uncover the basic parameters at the molecular, organismal, population, and community levels that regulate the degradative ability of specific microbial populations, the kinetics of degradations, and ecological constraints that modulate biodegradation in the environment. Components of this rather large research agenda are developing within the field and include investigations on structure-activity relationships in predicing biodegradation, comparative investigations on biodegradation kinetics among

diverse environmental matrices, and investigations on the molecular mechanism conferring degradative capacity on individual population of microorganisms. In addition to these developments in the field new contributions in the area of molecular microbial ecology may provide tools to study the structure and function of biodegradative microbial communities. Among these tools are new gene probe applications that can provide quantitative genotypic information on the occurrence, frequency, mobility, and evaluation of catabolic genes in microbial communities.

The objective of this report is to demonstrate the potential applications of gene probe technology in environmental biodegradation research. These applications include: (1) developing kinetic constants for biodegradation specific to those strains that are genotypically capable of biodegradation, (2) rapid enumeration and isolation of related catabolic phenotypes from environmental samples, (3) quantifying the maintenance and stability of catabolic genotypes in environmental samples, and (4) developing genetic control strategy for optimizing the catabolism of environmental contaminants.

Applications for gene probes can be coupled with conventional microbiological enumeration techniques, whereby discrete colonies serve as the source of target DNA to which a gene probe is directed. However, the potential also exists to extract total DNA from a microbial community and to use this DNA as the primary target to quantitatively determine DNA sequences homologous to specific probe DNA.

Four applications for DNA probes in biodegradation research were chosen to demonstrate their potential utility. The applications include:

(1) The use of whole NAH7 (naphthalene degradation) plasmid DNA probe in determining the biodegradative response of sediment microcosm populations to contamination by polyaromatic hydrocarbon rich, synthetic oils;

(2) The use of the 4-chlorobiphenyl (4CB) catabolic plasmid pSS50 as probe DNA to quantitate and isolate related 4CB catabolic phenotypes in environmental samples;

(3) The use of TOL (toluene/xylene degradation) specific and RK2 plasmid DNA probes to study the maintenance of P. putida (carrying pWWO and RK2) introduced into chemically contaminated groundwater aquifer material; and

(4) The use of a NAH specific DNA probe to measure NAH plasmid population dynamics in a lab scale industrial waste treatment process and NAH sequence frequency in full scale, municipal activated sludge.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Their Maintenance

Bacterial strains and plasmids used in this study are shown in Table 1. Strains carrying plasmids TOL (pWWO), NAH7, and pSS50 were maintained on minimal agar plates (5) containing m-toluate (300 mg l^{-1}), naphthalene (in vapor form), or 4-chlorobiphenyl (100 mg l^{-1}), respectively; minimal agar plates containing 4-chlorobiphenyl were supplemented with 0.001 g l^{-1} yeast extract to provide necessary amino acids and vitamins. P. putida carrying RK2 and E. coli strains carrying plasmids pTS206 or pDTG113 were maintained on yeast extract peptone glucose (YEPG) agar (17) containing kanamycin (Km, 100 ug ml^{-1}). Routine maintenance of strains was on YEPG agar or L-agar medium.

Plasmid pTS206 carries a 0.6 kb Bgl II fragment of the xyl R gene fragment of TOL plasmid cloned into pMCR600 (13). Plasmid pDTG113 contains an approximately 15 kb EcoRI fragment of NAH7 which is cloned into vector pKT230 (D. T. Gibson, personal communication); pDTG113 appears to carry the upper pathway genes of naphthalene degradative pathway. Descriptions of the plasmids TOL (pWWO), NAH7, pTS206, pDTG13, and the broad host range antibiotic resistant plasmid RK2 are given in Figures 1-3. The 4-CB catabolic plasmid pSS50 is not fully characterized but is a transmissible 53.2 kb plasmid in Alcaligenes sp. and shows no hybridization with other common catabolic plasmids thus far examined (6, 20, Sayler et al., unpublished).

Bacterial Enumeration and Microcosm Preparation

Enumeration of aerobic heterotrophic bacteria was by spread plate inoculation of 1/10 strength YEPG agar. Sediment or aquifer samples were diluted 100 fold in phosphate buffer (pH 7.0) and mixed to dislodge organisms; serial dilution were prepared in phosphate buffered saline prior to inoculation of the appropriate medium.

Microcosm sediment samples exposed to synthetic oil contamination have been previously described (18). These sediments were challenged with various concentrations of ^{14}C-naphthalene and ^{14}C-phenanthrene to measure kinetics of biodegradation based on the evolution of $^{14}CO_2$ as previously described (18). Pristine groundwater aquifer materials were challenged with a mixture of toluene, styrene and chlorobenzene in a trichloroethylene carrier (400 ug l^{-1} each component) to exert environmental selective pressure on the maintenance of TOL plasmid DNA in P. putida (TOL and RK2) introduced into these samples (8).

DNA Isolation and Purification

Plasmid DNA was isolated by modification of the alkaline lysis procedure of Kado and Liu (9) (NAH7), the lysozyme-SDS procedure of Anderson and McKay (1) (pSS50) or the Triton X-100 cleared lysate procedure (10) (pTS206 and pDTG113). Purification of plasmid DNA in above cases was accomplished by dye-buoyant density ultracentrifugation in cesium chloride/ethidium bromide at 40,000 rpm for up to 48 hr using a Beckman Ti50 rotor and Beckman L8-80 Ultracentrifuge. Where needed, plasmids were examined by agarose gel electrophoresis using 0.7% to 0.8% agarose vertical slab gels as previously described (20).

Activated sludge total DNA was isolated from 1 liter samples of activated sludge obtained from a municipal wastewater treatment plant with major modification of the Torsvik's method (22). The cell mass was harvested by centrifugation and washed with 0.12 M phosphate buffer, pH 8.0 to remove loosely associated humic materials. The washed cells were resuspended in 0.12 M phosphate buffer, pH 6.8, and incubated with lysozyme (Sigma) at 4.0 mg ml^{-1} at 37°C for 1 hour. The lysozyme treated sample was brought to 2.5% SDS and treated with proteinase K (Sigma), 1.0 mg ml^{-1}, for 1 hr at 65°C to remove

the proteins from the sample. The sample was then subjected to glass bead (siliconized) homogenization in a blender for 5 min.

The lysed cell suspension was subjected to a clarifying centrifugation at 20,000 rpm and the aqueous phase decanted and extracted to completion with phenol:chloroform:isoamyl alcohol (50:50:1) (7 to 13 total extractions) and re-extracted with chloroform:isoamyl alcohol and the DNA was precipitated by ethanol precipitation at -20°C. The precipitated DNA was resuspended in 0.12 M phosphate buffer and was treated with RNase (RNase A, pancreatic), 50 mg ml^{-1} for 30 min. at 37°C in order to avoid the RNA contamination. The RNase treated samples were re-extracted with phenol: chloroform:isoamyl alcohol and re-precipitated with ethanol. The recovered DNA was suspended in 0.12 M phosphate buffer, pH 6.8, and reconcentrated using Centricon tubes when needed.

To further remove the contaminating humic-like materials the concentrated DNA samples were applied in 10 ml volumes to a 60 ml DEAE-Sepharose column (3.6 cm bed diameter) and eluted with 0.7 M KCl-10 M Tris HCl buffer, pH 7.3. Eluants containing DNA were combined and mixed with 20 ml of hydroxylapatite for 1 hr and the mixed sample was poured into siliconized glass columns. The column was eluted with 30 ml of 8 M urea, 0.24 M phosphate buffer and then 150 ml of 0.014 M sodium phosphate. The purified DNA was then eluted off the hydroxylapatite with 0.4 M sodium phosphate buffer, pH 8. DNA purity and relative molecular weight was determined by UV absorbance ratios (280:260 nm) and agarose gel electrophoresis, respectively. The approximate size of the purified heterogeneous DNA from activated sludge was approximately 4.0 kb.

DNA Probe Preparation and Hybridization

Detection and quantitation of target DNA, homologous to sequences of individual probe DNA, was performed via hybridization with ^{32}P-labeled probe DNA followed by autoradiography. This approach was used for direct screening of

bacterial colonies or for direct probing of samples from total DNA extracts. ^{32}P-DNA probes were prepared by nick translation of purified plasmid DNA, as specified by the commercial suppliers of a nick translation reagent kit (Bethesda Research Laboratories, Gaithersburg, MD). ^{32}P-dCTP (New England Nuclear), was used as the sole labeling nucleotide and the labeled probe was purified by Sephadex G-50 column chromatography as previously described (19). Specific activity of the labeled DNA probes was generally in the range of 10^7 to 10^8 dpm ug^{-1} DNA.

Detection of target DNA sequences in bacterial colonies on enumeration plates or isolated cultures was by DNA:DNA colony hybridization (4) with the appropriate DNA probes. This application to environmental microbiology has been previously described (4, 16, 19). The general procedure for colony hybridization is outlined in Figure 4. Bacterial colonies are transferred to a hybridization membrane (nylon or nitrocellulose) and the cells are alkaline lysed and the denatured DNA (target) is thermally fixed in the single-stranded form to the hybridization membrane. Following a prehybridization treatment to eliminate nonspecific background, the ^{32}P-labeled boiled (single-stranded) probe DNA is added and, under permissive conditions, allowed to hybridize to homologous target sequences. A low salt (10 mM NaCl) wash buffer is used to disrupt hybrids of greater than 5% base pair mismatch (95% stringency), and the positive target colonies are detected by autoradiography.

Detection of target sequences in DNA extracts followed the same strategy, except that aliquots of appropriate concentrations of DNA (in solution) were directly blotted onto a hybridization membrane and fixed. Hybridization of the ^{32}P-labeled probe to homologous target sequences in the DNA blot is performed in an identical manner to that described for DNA:DNA colony hybridization.

RESULTS AND DISCUSSION

Kinetics of Naphthalene Mineralization

In initial studies on the oxidation of naphthalene in sediments, it was demonstrated that naphthalene mineralization could be described by a first order rate process (in substrate) (Fig. 5). Over a naphthalene concentration range of 10 ug l^{-1} to 100 mg l^{-1} mineralization was linear with respect to concentration. Mineralization rate constants in these previously uncontaminated sediments ranged from 3.9×10^{-5} hr^{-1} to 3.7×10^{-4} hr^{-1} in incubation conditions ranging from 3 to 30 days.

For sediments exposed to synthetic oil contamination for a period of time in excess of two years, both total viable cells and naphthalene mineralization kinetics were elevated approximately 20 fold over uncontaminated sediments (18). This community response was hypothesized to be due to a general enrichment of organisms capable of oxidation of aromatic hydrocarbons. This hypothesis was proven by demonstrating parallel increase in organisms capable of growth on naphthalene minimal medium (Table 2) and detection of higher proportions of NAH plasmid positive genotypes in synthetic oil contaminated sediments as compared to untreated control sediments (enumeration demonstrated by autoradiogram in Fig. 6).

Using the frequency of NAH^+ genotypes, second order rate constants (k_2) (first order in substrate and first order in cell number) were calculated to compare the contaminated and uncontaminated sediments (Table 2). In general, second order rate constants calculated on NAH^+ genotypes are greater than comparable estimates based on total viable cells or those from growth on naphthalene plates. These kinetic constants vary in accordance with the proportion of NAH^+ genotypes on the respective enumeration medium. Although the results for synthetic oil contaminated sediment appear consistent as k_2

values range from 3.9×10^{-13} to 9.0×10^{-13} organisms hr^{-1}; however, comparable k_2 values in uncontaminated sediments range from 8.5×10^{-13} to 4.1×10^{-11} organisms hr^{-1}. This variability and the elevated k_2 values (on a per organismal basis) are unexplained but may be related to the diversity of genotypes in uncontaminated sediment and the lack of probe specificity; whole NAH7 plasmid DNA, which shares homology with other catabolic plasmids such as TOL as indicated in figure 1, was used as probe. These limitations make absolute estimates of k_2 values for mineralization of naphthalene impossible at this time, but the results do suggest an approach for developing more accurate predictors for the kinetics of aromatic hydrocarbon biodegradation.

Detection and Isolation of 4-Chlorobiphenyl Degradative Phenotypes

The 53.2 kb, 4-chlorobiphenyl mineralization plasmid pSS50 was used as a whole plasmid probe to isolate related catabolic genotypes in polychlorinated biphenyl contaminated reservoir sediments. Using YEPG agar medium for enumeration of total viable cells in sediments, approximately 0.3% of the total sediment cell forming unit (CFU) were received at demonstrating positive homology with pSS50 (16). Random isolates were selected from replica plates and were screened and confirmed for their ability to produce 4-chlorobenzoate as a metabolite during the biodegradation of 4-chlorobiphenyl (16). Three unidentified isolates, LPS9, LPS9B, and LPS9C (Table 3), were selected for further study on their ability to grow on and degrade other chlorobiphenyl substrates.

As indicated by Table 3, reservoir isolates LPS 9, 9AB, and 9C along with the original Alcaligenes sp. A5 host strain for pSS50, demonstrated the ability for growth on 4-; 4,4'-; 2,4,5,- and 2,4,2',4'-chlorobiphenyl. Partial characterization of the biodegradation of 4-chloro and 4,4'-dichlorobiphenyl by these new isolates, obtained by colony hybridization, demonstrates that they are

proficient at significant degradation of the substrates with concomitant production of the corresponding 4-chlorobenzoic acid as a transient degradation product (Table 4).

These new isolates capable of chlorobiphenyl degradation were recovered in approximately one week time from the point of field sampling to isolation of pSS50 positive colonies from replica plates. This approach using DNA:DNA colony hybridization, avoided long term selective enrichment for degradative organisms and permitted the rapid isolation and screening of a variety of sediment isolates all of which were confirmed to degrade some chlorobiphenyl substrates.

Maintenance of P. putida (TOL and RK2) in Groundwater Aquifer Material

P. putida was introduced into pristine and experimentally contaminated groundwater aquifer material obtained from a field site in Lula, OK. Colony hybridization using plasmids RK2 and pTS206 (carrying xyl R fragment of TOL plasmid, pWWO), were used as gene probes to study the maintenance and stability of this genetically-modified model organism.

To monitor the maintenance of P. putida (TOL and RK2) colony hybridization was used in conjunction with YEPG, m-toluate and YEPG-Km enumeration agar media. As demonstrated in Figure 7, both TOL and RK2 were stably maintained at a frequency of approximately 1×10^5 g^{-1} aquifer material following an eight week incubation of P. putida (TOL and RK2) introduced in non-sterile aquifer material. The three enumeration media appeared differential in their ability to select for strains harboring both plasmids. This is most clearly evident by comparing the TOL and RK2 positive hybrids recovered on YEPG-Km agar medium which appears to counter select against TOL harboring strains (Fig. 7). TOL^+ genotypes on this medium appeared to be maintained at 1×10^3 organisms g^{-1}, approximately two orders of magnitude lower than that recovered for either TOL or RK2 on the remaining media. Consequently, this lowered

maintenance is actually an artifact of the selection for TOL^-, $RK2^+$ strains during growth on YEPG-Km agar medium during enumeration.

Random isolates were selected from each medium type and were examined for the presence of the TOL and RK2 plasmids using agarose gel electrophoresis. As indicated by Figure 8A, both TOL and RK2 were recovered by enumeration on the m-toluate minimal agar medium. However, TOL and RK2 were selected independently by enumeration on YEPG agar and YEPG-Km agar media, respectively; the frequency of loss of TOL and RK2 was up to 10% on YEPG-Km agar and YEPG agar media, respectively and consequently, only one or the other plasmid was detected in these sub-populations (Fig. 8B and 8C). These results confirm the observation made by colony hybridization alone. Overall these results demonstrate that a genetically-modified model organism can be stably maintained in groundwater aquifer material and that contaminant selection played no significant role in this stable maintenance, while the enumeration medium imposes as severe selective pressure.

NAH Plasmid Population Dynamics in a Lab Scale Industrial Waste Treatment Process

A continuous lab scale activated sludge bioreactor was established using an industrial wastewater (rich in aromatic hydrocarbons and acrylates) as the feed stock and industrial sludge as seed for the bioreactor (a detailed study describing the equipment design and other aspects of the bioreactor performance will be published elsewhere; manuscript in preparation). The fate of naphthalene was tracked using a ^{14}C-naphthalene as a tracer, and both conventional microbiological enumeration of bacterial populations and gene probe analysis were employed to monitor bacterial and NAH plasmid population dynamics. The recombinant plasmid pDGT113 containing an approximately 15 kb insert of the

NAH7 plasmid (encoding upper pathway; Gibson, unpublished) served as a specific probe for the naphthalene-degrading population.

During the course of more than three week experimental run, stripping and biodegradation were the predominant fates of naphthalene in the bioreactor (Fig. 9A). Mineralization and stripping of naphthalene were inversely related, with up to 80% of the naphthalene being mineralized during optimum reactor performance. Total viable cells based on enumeration on YEPG agar medium were maintained at a range of approximately 10^9 cells ml^{-1} and were an insensitive indicators of naphthalene degradative performance (Fig. 9B); the bacterial populations capable of growth on naphthalene minimal agar medium were maintained at approximately 10^6 cells ml^{-1}. Also, as indicated by Figure 9B, NAH^+ genotypes as estimated from probing YEPG grown colonies were approximately an order of magnitude greater than naphthalene degraders by plate count further showing the sensitivity and significance of gene-probe technology. In addition, major increases in NAH^+ frequency faithfully reflected bioreactor performance in terms of naphthalene mineralization (comparison of Fig. 9A and B).

These results suggest that monitoring of specific catabolic genotypes within the heterogeneous activated sludge community is feasible and could be used to predict the performance of a waste treatment process. In addition, if appropriate system control can be developed to optimize population densities of NAH^+ genotypes, it should be possible to optimize bioreactor performance for biodegradation of specific components within a waste stream.

Detection of NAH Catabolic Genes in Activated Sludge Total DNA

A new approach for measuring the concentration or frequency of catabolic genes in heterogeneous microbial communities is the extraction of total DNA from the community and directly probing in a quantitative manner for the presence of specific genes or plasmids. Potentially, this approach would avoid the

limitations of culturing organisms from samples and would speed the analysis of specific genotypes.

In initial experiments, aliquots of sludge DNA from 10 ug to .001 ug were blotted onto hybridization membrane and probed with NAH7 plasmid DNA (Fig. 10). Positive hybridization signals were detected at levels down to 0.5 ug total DNA suggesting the presence of NAH7 homologous sequences in the extracted sludge DNA. As a positive control (lane D, Fig. 10) .05 to .0000005 ug of NAH7 DNA was added to the sludge DNA; this DNA was detected by hybridization down to .0005 ug of NAH7 DNA which was added to .25 ug total sludge DNA. Basically, total genomic DNA from a gram-positive bacteria (B. cereus; lane A) and a gram-negative bacteria (E. coli; lane B), which were used as negative controls, did not appear to show any homology with the NAH7 DNA used as probe; however, a very weak positive hybridization signal was detected in the case of E. coli (only when 10 ug DNA was blotted). Judging from the intensity of NAH7 DNA probed with itself (lane E, Fig. 10) and the NAH7 plus sludge positive control (lane D), approximately .00005 ug of $NAH7^+$ sequence homology was detected in .25 ug of total sludge DNA. This concentration of NAH7 related sequences represents a relative abundance 0.020% of the total sludge DNA.

The utility of this approach is that it may be possible to achieve quantitative measures (depending upon gene probe specificity) of specific genotypes without cultivation of organisms from environmental samples. Such an approach could accomplish the same result as colony hybridization but it would add speed to the procedure. More importantly, this approach would be independent of the ability of organisms to grow on a cultivation medium that may arbitrarily select against its survival (as demonstrated for TOL, Fig. 7).

Strengths and Limitations of Gene Probe Applications

In applying gene probe techniques to the quantitative enumeration of biodegradative genotypes it is necessary to be aware of factors effecting the

accuracy and interpretation of the results obtained. In colony hybridization approaches, the method, for better or worse, is tied to the conventional cultivation of environmental populations. Such cultivation and enumeration is known to be highly selective and generally capable of only recovering a fraction of total populations in a given habitat. Using the colony hybridization approach, only those organisms capable of producing at least a microcolony can be examined as targets for a specific DNA probe. Secondly, the lysis of colonies preliminary to hybridization may need to be varied or supported by additional lysis treatments. Consequently alkaline lysis may not be appropriate for all colonies and may need inclusion of alternate treatment such as lysozyme-SDS; lysis should be monitored by visual observation or dye treatment (personal communication, B. Olsen, University of California, Irvine).

In addition to these procedural problems, there is an absolute need to be knowledgeable of the specificity and cross reactivity of the specific probe used. This is true for probes derived for whole, native plasmid, inserts in cloning vectors, and chromosomal or cloned chromosomal probes. The ancestory of the cloning vector itself is important in determining specificity and can cloud results. However, vector DNA itself can be used in prehybridization to reduce any background associated with vector hybridization.

Given these limitations, probes application can provide a new generation of genotype specific information in biodegradative research. Developing information suggests that application of colony hybridization using nonselective growth medium may provide the greatest amount of genotypic information on total populations contributing immunity or consortia metabolism of organic molecules.

An exciting new possibility is evaluating genotype frequency in total DNA of a microbial community. Such an approach would avoid most of the limitations

associated with cultivation and required growth of organisms isolated from the environment. Future developments of non-isotopically labeled probes may also make in situ application possible for some environmental matrices. These and future applications for DNA or RNA probes can provide important ecological insight into the biodegradative potential of microbial communities and will contribute significant information of the efficacy and safety of organisms containing recombinant genes developed for enhanced biodegradative potential.

ACKNOWLEDGEMENTS

This research was supported by U.S. Environmental Protection Agencies Cooperative agreements CR810485, R.S. Kerr Environmental Research Laboratory and CR812488, Gulf Breeze Environmental Research Laboratory, by training grant T32 AI 07123 National Institutes of Health, U.S. Public Health Service and a grant from The University of Tennessee Hazardous Waste Institute.

REFERENCES

1. Anderson, D. G., and L. L. McKay. 1983. Simple and rapid method for isolating large plasmid DNA from lactic streptococci. Appl. Environ. Microbiol. 46:549-552.

2. Franklin, F. C. H., M. Bagdasarian, M. M. Bagdasarian, and K. N. Timmis. 1981. Molecular and functional analysis of the TOL plasmid pWWO from Pseudomonas putida and cloning of genes for the entire regulated aromatic ring meta cleavage pathway. Proc. Natl. Acad. Sci. U.S.A. 78:7458-7462.

3. Grund, A. D. and I. C. Gunsalus. 1983. Cloning of genes for naphthalene metabolism in Pseudomonas putida. J. Bacteriol. 156:89-94.

4. Hanahan, D., and M. Meselson. 1980. Plasmid screening at high colony density. Gene 10:63-67.

5. Hegeman, G. D. 1966. Synthesis of the enzymes of the mandelate pathway in Pseudomonas putida. I. Synthesis of enzymes by the wild type. J. Bacteriol. 91:1140-1154.

6. Hooper, S. W., and G. S. Sayler. Characterization of the 4-chlorobiphenyl mineralization plasmid, pSS50. Plasmid (submitted).

7. Inouye, S., A. Nakazawa, and T. Nakazawa. 1985. Determination of the transcription initiation site and identification of the protein product of the regulatory gene xyl R for xyl operons on the TOL plasmid. J. Bacteriol. 163:863-869.

8. Jain, R. K., G. S. Sayler, J. T. Wilson, L. Houston, and D. Pacia. 1986. Maintenance and stability of introduced genotypes in groundwater aquifer microcosms. Appl. Environ. Microbiol. (submitted).

9. Kado, C. I., and S.-T. Liu. 1981. Rapid method for detection and isolation of large and small plasmids. J. Bacteriol. 145:1365-1373.

10. Kahn, M., R. Kilter, C. Thomas, D. Figurski, R. Meyer, E. Remaut, and D. R. Helinski. 1979. Plasmid cloning vehicles derived from plasmids Col El, F, R6K, and RK2, vol. 88, pp. 208-280. In R. Wu (ed.), Methods in Enzymology. Academic Press, Inc., New York.

11. Lehrbach, P. R., I. McGregor, J. M. Ward, and P. Broda. 1983. Molecular relationships between Pseudomonas INC P-9 degradative plasmids TOL, NAH, and SAL. Plasmid 10:164-174.

12. Maniatis, T., F. Fritsch, and J. Sambrook. 1982. Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

13. Nakazawa, T., S. Inouye, and A. Nakazawa. 1985. Positive regulation and transcription initiation of xyl operons on TOL plasmid, p. 415-429. In D. R. Helinski, S. N. Cohen, D. B. Clewell, D. A. Jackson, and A. Hollaender (eds.), Plasmids in Bacteria. Plenum Press, New York.

14. Miki, T., H. Kumahara, and A. Nakazawa. 1981. Construction of a fused operon consisting of the rec A and kan (kanamycin resistance) genes and regulation of its expression by the lex A gene. Molec. Gen. Genet. 183:25-31.

15. Nakazawa, T., and T. Yokota. 1977. Isolation of a mutant TOL plasmid with increased activity and transmissibility from Pseudomonas putida (arvilla) mt-2. J. Bacteriol. 129:39-46.

16. Pettigrew, C. A. and G. S. Sayler. 1986. The use of DNA-DNA colony hybridization in the rapid isolation of 4-chlorobiphenyl degradative bacterial phenotypes. J. Microbiol. Methods (In press).

17. Sayler, G. S., L. C. Lund, M. P. Shiaris, T. W. Sherrill, and R. E. Perkins. 1979. Comparative effects of Aroclor 1254 (polychlorinated biphenyls) and phenanthrene on glucose uptake by freshwater microbial populations. Appl. Environ. Microbiol. 37:878-885.

18. Sayler, G. S., R. E. Perkins, T. W. Sherrill, B. K. Perkins, M. C. Reid, M. S. Shields, H.-L. Kong, and J. W. Davis. 1983. Microcosm evaluation of microbial community response to synthetic oil contamination in freshwater sediments. Appl. Environ. Microbiol. 46:211-219.

19. Sayler, G. S., M. S. Shields, E. T. Tedford, A. Breen, S. W. Hooper, K. M. Sirotkin, and J. W. Davis. 1985. Application of DNA-DNA colony hybridization to the detection of catabolic genotypes in environmental samples. Appl. Environ. Microbiol. 49:1295-1303.

20. Shields, M. S., S. W. Hooper, and G. S. Sayler. 1985. Plasmid-mediated mineralization of 4-chlorobiphenyl. J. Bacteriol. 163:882-887.

21. Thomas, C. M., C. A. Smith, V. Shingler, M. A. Cross, A. A. K. Aussian, and M. Pinkney. 1985. Regulation of replication and maintenance functions of broad-host-range plasmid RK2, pp. 261-276. In D. R. Helinski, S. W. Cohen, D. B. Clewell, D. A. Jackson, and A. Hollaender (eds.), Plasmids in Bacteria. Plenum Press, New York.

22. Torsvik, V. L. 1980. Isolation of bacterial DNA from soil. Soil Biol. Biochem. 12:15-21.

23. Yen, K.-M. and I. C. Gunsalus. 1982. Plasmid gene organization: Naphthalene/salicylate oxidation. Proc. Natl. Acad. Sci. U.S.A. 79:874-878.

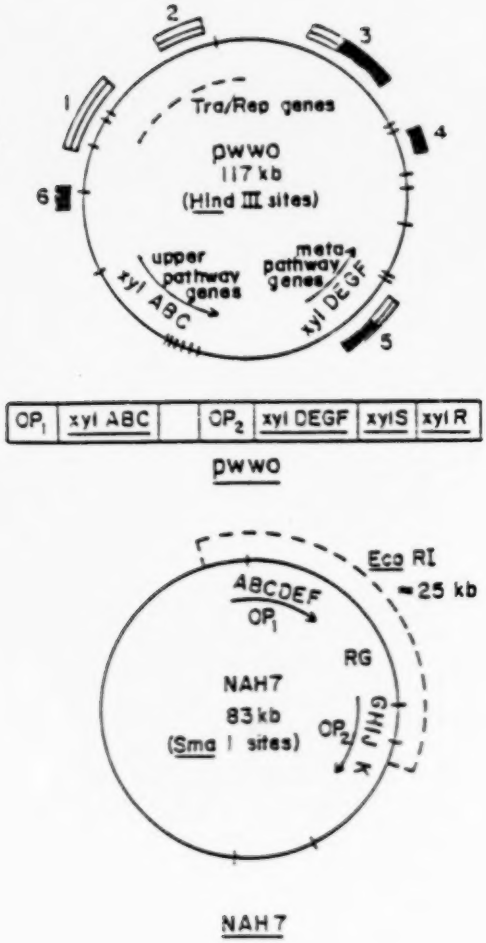


Figure 1. Simplified physical and functional maps of pWWO (TOL) and NAH7. Map of pWWO has been adapted from Franklin et al. (1981), Lehrbach et al. (1983) and Nakazawa et al. (1985); Tra/Rep denote the determinants of autonomous conjugal transfer and replication functions. Regions (1 to 6) showing relatively strong (▬) or weak (▬) hybridization to pWWO DNA with ^{32}P-labeled NAH7 DNA. The bar diagram represents the location of toluene/xylene degradative genes. Op_1, operon one and Op_2, operon two. xyl ABC represent the genes of upper pathway and xyl DEGF represent the genes of lower pathway; xyl R and xyl S are the regulatory genes of the pathway.

Map of NAH7 was adapted from Yen and Gunsalus (1982) and Grund and Gunsalus (1983); Op_1 and Op_2 represent two operons and RG represent regulatory genes separating two clusters of genes-ABCDEF and GHIJK.

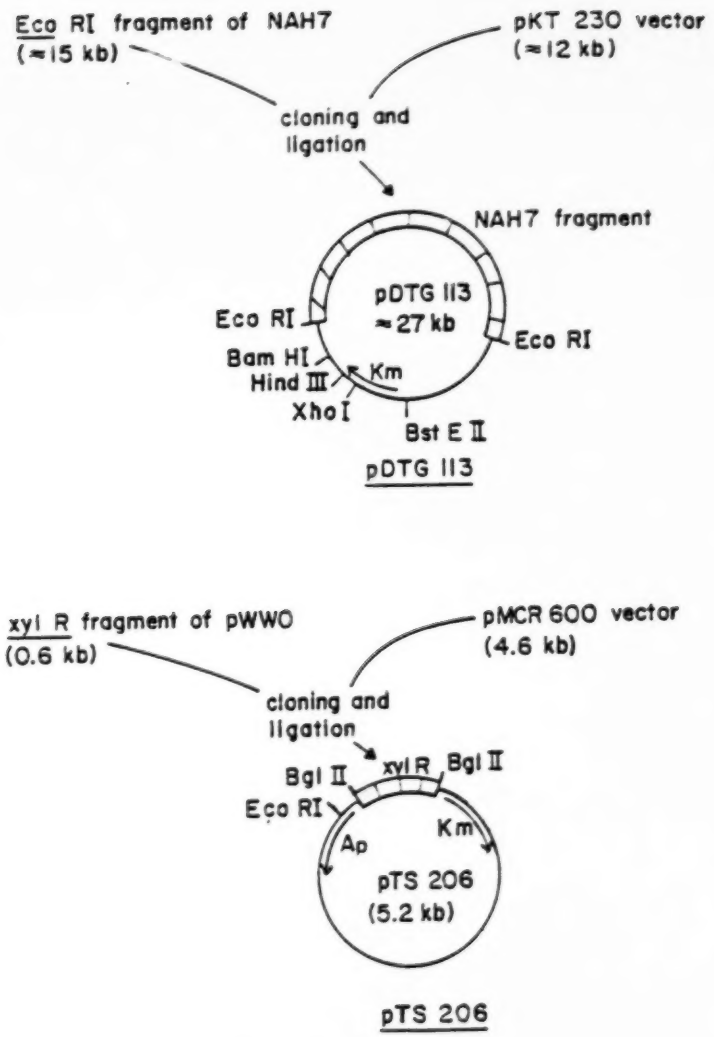
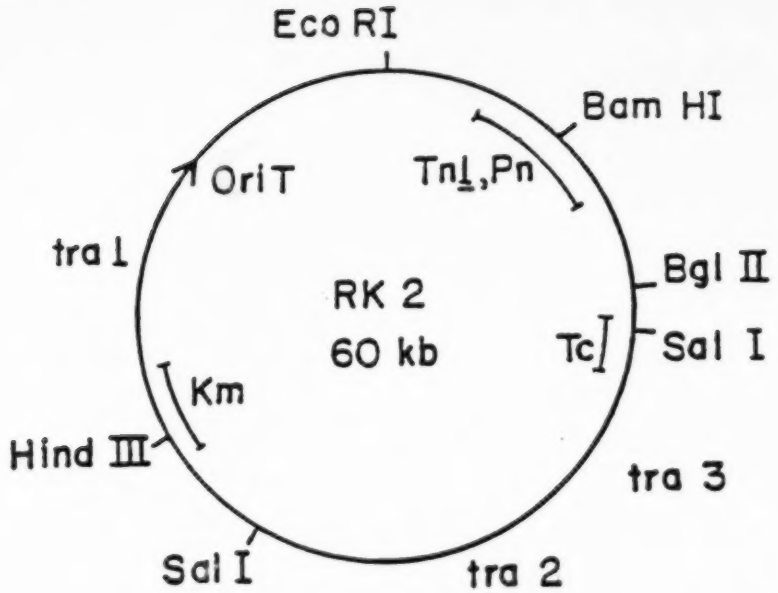


Figure 2. Constructions of pDTG113 and pTS206. Construction of pDTG113 has been adapted from Gibson (unpublished); Km, resistance to kanamycin. Construction of pTS206 has been adapted from Miki et al. (1981) and Inouye et al. (1985); Ap, resistance to ampicillin and Km, resistance to kanamycin.



RK 2

Figure 3. Simplified physical and genetic map of RK2 (adapted from Thomas et al., 1985). Some of the symbols are specified as follows: Pn^r, penicillin resistance marker(s); Tc^r, tetracycline resistance marker(s); Km^r, kanamycin resistance marker(s); ori T, origin of transfer.

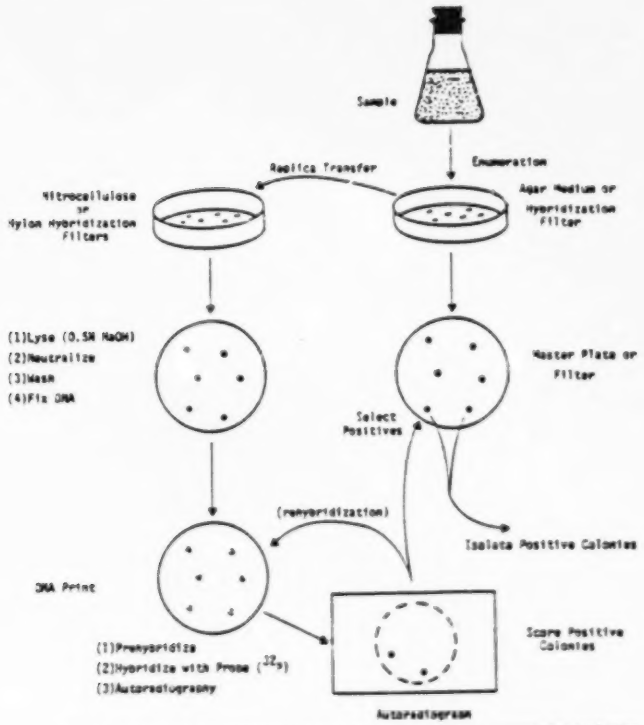


Figure 1. Schematic representation of the colony hybridization approach for isolation of catabolic phenotypes in environmental samples.

Figure 4. General procedure for the application of DNA:DNA colony hybridization for the detection and isolation of bacterial phenotypes from environmental samples.

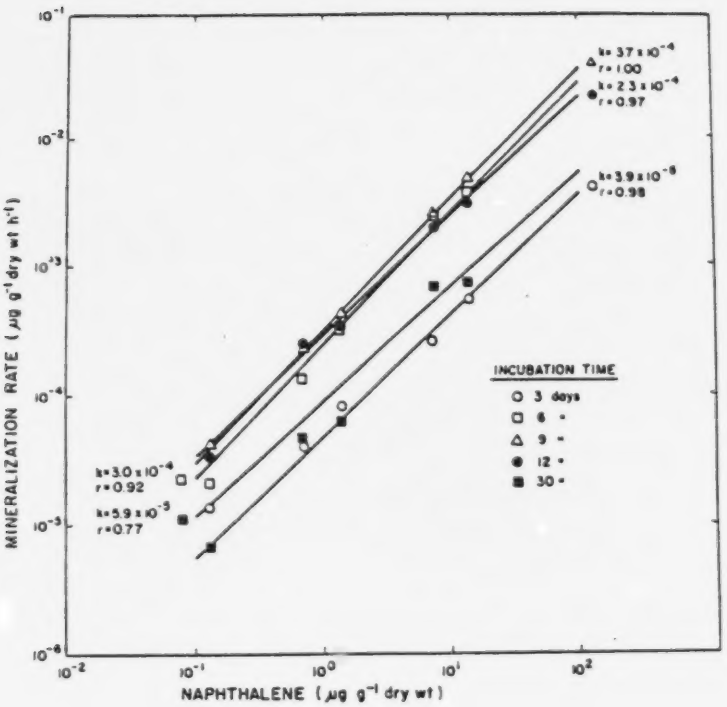

MINERALIZATION RATE (μg g⁻¹ dry wt h⁻¹)
NAPHTHALENE (μg g⁻¹ dry wt)
INCUBATION TIME
3 days
6 "
9 "
12 "
30 "
k= 3.7 x 10⁻⁴ r= 1.00
k= 2.3 x 10⁻⁴ r= 0.97
k= 3.9 x 10⁻⁵ r= 0.98
k= 3.0 x 10⁻⁴ r= 0.92
k= 5.9 x 10⁻⁵ r= 0.77

Figure 5. Relationship between naphthalene mineralization rate and substrate concentration in freshwater sediment (uncontaminated sediment treated with ^{14}C-naphthalene and incubated for varying time intervals. Mineralization determined as $^{14}CO_2$ evolution. k = 1st order mineralization rate constant with respect to substrate).

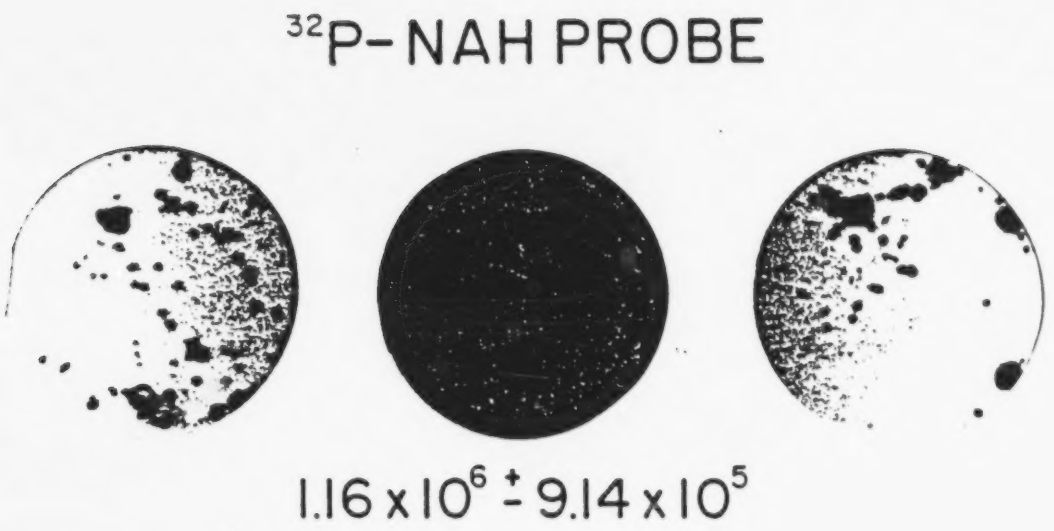


Figure 6. Autoradiographic detection of NAH7$^+$ genotypes in bacterial populations recovered from synthetic oil contaminated sediments (YEPG agar enumeration medium, colonies probed by DNA:DNA hybridization using labeled ^{32}p-NAH7 plasmid DNA).

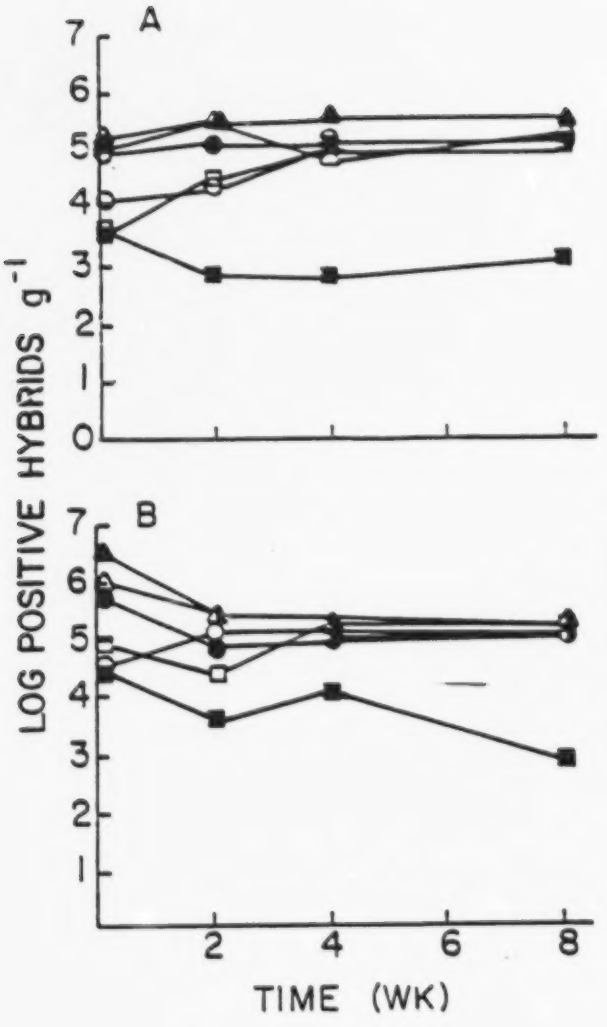


Figure 7. Comparative selection of TOL and RK2 in P. putida (TOL and RK2) inoculated groundwater aquifer microcosms; A, uncontaminated and B, chemically contaminated. ● , ▲ and ■ represent the density of TOL sub-population using as selection medium either YEPG agar, m-toluate minimal agar or YEPG agar supplemented with Km (100 ug ml^{-1}), respectively; pTS 206 was used as the probe DNA. ○ , △ and □ represent the density of RK2 sub-population using as selection medium either YEPG, agar, m-toluate, minimal agar or YEPG agar supplemented with Km (100 ug ml^{-1}), respectively; RK2 was used as the probe DNA.

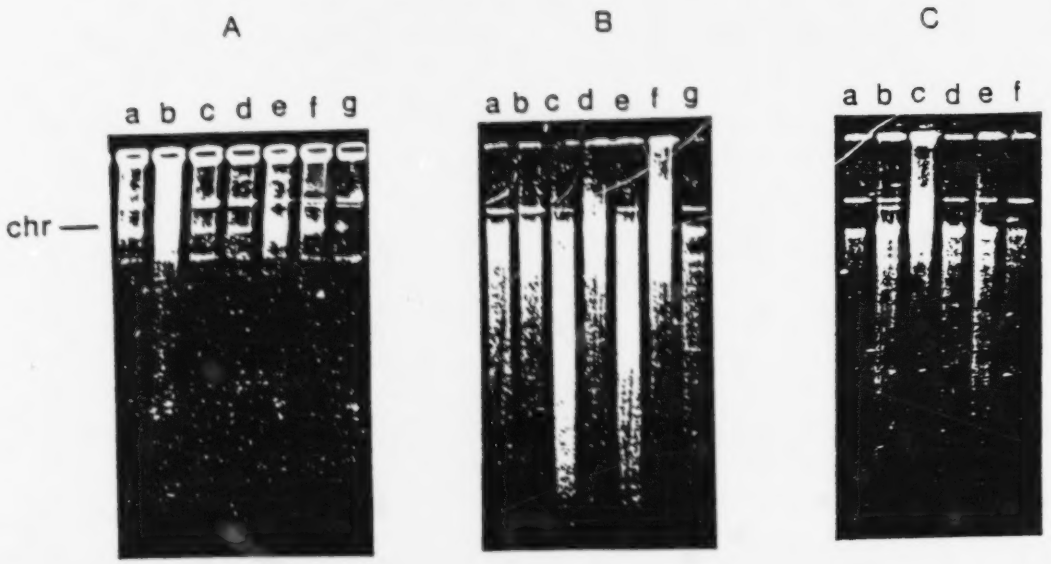


Figure 8. Agarose gel electrophoresis (0.7% w/v, using TAE as buffer) of plasmid DNA isolated by the method of Kado and Liu (9). A. a, P. putida carrying plasmids TOL and RK2 as a standard, b-g, colonies picked from m-toluate minimal agar. B. a, E. coli carrying RK2 as a standard, b-g, colonies picked from YEPG agar medium containing Km. C; b, P. putida carrying plasmids TOL and RK2 as a standard, a and c-f, colonies picked from YEPG agar medium. The lower most band in figures A and B is the linear form of RK2.

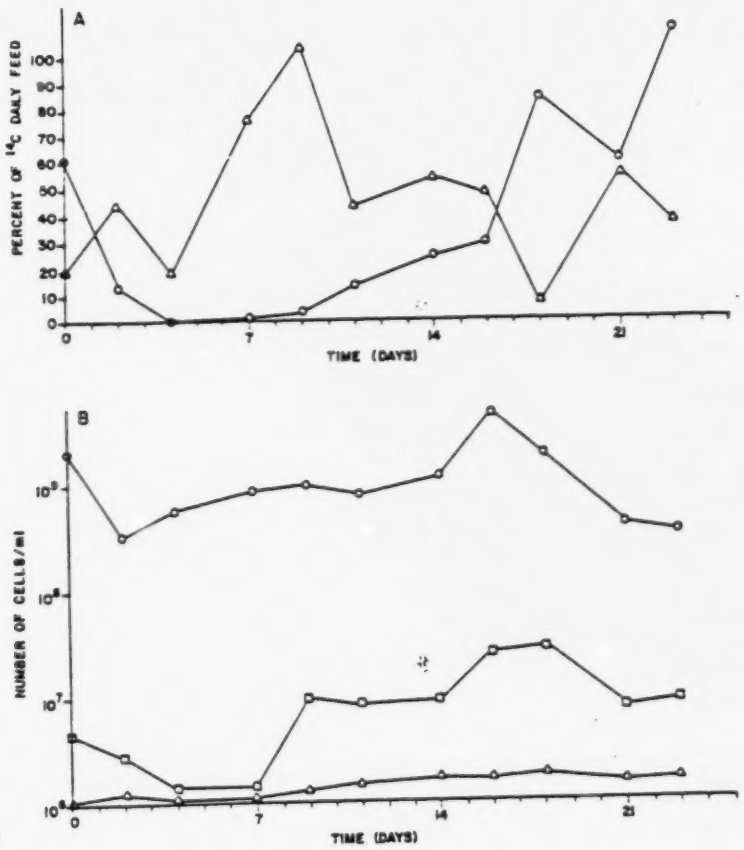


Figure 9. Naphthalene biodegradation and population dynamics of a continuous mixed culture reactor over a 3-4 wk period fed with ^{14}C-naphthalene. Figure A shows the mineralization rate (○) and stripping rate (△) of naphthalene. Figure B shows the total number of viable cells (○) on YEPG agar plates, total number of naphthalene degrading cells (□) as determined by the gene-probe analysis (see text) and total number of naphthalene degrading cells (△) as determined by the naphthalene minimal agar plate counts.

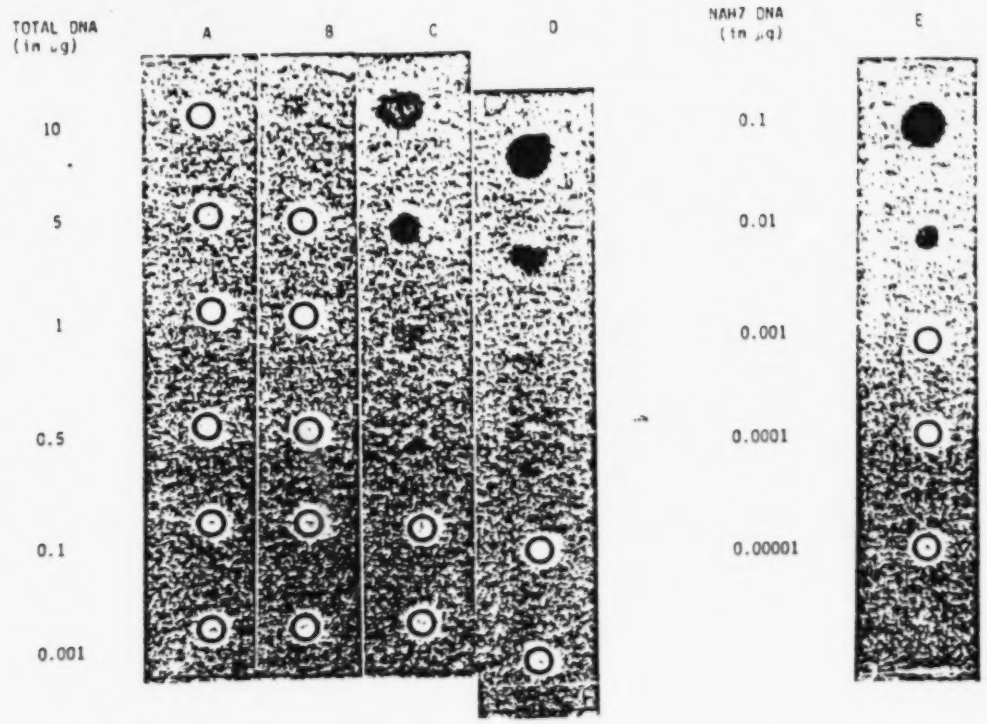


Figure 10. Detection of the NAH7^{+} catabolic genes in activated sludge DNA. Total NAH7 (^{32}P) DNA was used as probe DNA. Lanes A, Bacillus cereus chromosomal DNA as negative control; B, E. coli chromosomal DNA as negative control; C, total sludge DNA (see methods); D, total sludge DNA and NAH7 DNA together; E, NAH7 DNA only as positive control. DNA concentrations are as shown on the figure except in lane D where concentrations (in ug) are 5 (sludge) + 0.05 (NAH7), 2.5 (sludge) + .05 (NAH7), 0.5 (sludge) + .0005 (NAH7), 0.25 (sludge) + .00005 (NAH7), .05 (sludge) + .000005 (NAH7), and .0005 (sludge) + .0000005 (NAH7), respectively. The encircled areas did not show any homology or was below detection limits.

Table 1. Bacterial strains and plasmids used in this study

Strain	Plasmid complement(s) (size in kilobase pairs)	Source or reference
P. putida (arvilla) mt-2	TOL or pWWO (117)	15
P. putida	NAH7 (83)	23
Alcaligenes sp. A5	pSS50	20
Alcaligenes sp. A5	-	20
E. coli PRC 116	RK2 (58)	21
P. putida (arvilla) mt-2	RK2 and TOL (58 and 117)	8
E. coli C600	pTS206 (5.2)	7
E. coli BHB 2600	pDTG113 (app. 27)	D. T. Gibson
Bacillus cereus ATCC 13061	-	ATCC
E. coli DH1	-	12

Table 2. Comparative naphthalene mineralization kinetics in synthetic oil contaminated sediments

Synthetic oil treatment	Naphthalene mineralization k (hr^{-1})	Enumeration medium	CFU		Naphthalene mineralization	
			Total ml^{-1}	NAH^+ genotype	k_2 (organisms hr^{-1}) Total	NAH^+ genotype
None	4.5×10^{-4}	YEPG	5.3×10^5	50.9%	8.5×10^{-13}	1.7×10^{-12}
		Naphthalene	2.0×10^5	.05%	2.2×10^{-12}	4.1×10^{-11}
1% Synthetic oil	7.3×10^{-3}	YEPG	1.9×10^7	70.8%	3.9×10^{-13}	5.6×10^{-13}
		Naphthalene	1.1×10^7	73.6%	6.6×10^{-13}	9.0×10^{-13}

CFU, cell forming unit

Table 3. Growth of reservoir isolates, obtained by pSS50 colony hybridization, on chlorobiphenyls

Strain	Growth on 4-CB[a]	Growth on 4,4'-CB[a]	Growth on 2,4,5-CB[a]	Growth on 2,4,2',4'-CB[a]
LPS9	+++	+++	++	+
LPS9AB	+	+	+	+
LPS9C	+++ (y)[b]	+++ (y)[b]	+	+
A5	+++ (y)[b]	+++ (y)[b]	+	+
A5 (pSS50$^-$)	-	-	-	-

[a]Ten day incubation at 100 mgl^{-1} of chlorinated biphenyl substrate as indicated.

[b]Cultures turn bright yellow, suggesting the formation of high concentrations of the ring fission product.

Table 4. Biodegradation of 4-chlorobiphenyl and 4,4'-dichlorobiphenyl by bacterial isolates obtained by colony hybridization with a pSS50 plasmid probe

Strain	4-Chlorobiphenyl[a]		4,4'-Dichlorobiphenyl[b]	
	Removal (%)	4-CBA[c] detection (mg l^{-1})	Removal (%)	4-CBA[b] detection (mg l^{-1})
LPS9	94.4	16.43	58.7	5.13
LPS9AB	34.3	0.29	29.7	0.16
LPS9C	91.1	10.90	53.1	4.15
A5 control	97.9	19.97	58.0	4.12
A5 ($pSS50^-$)	33.5	0	-	-
Sterile control[d]	17.8	0	18.2	0

[a]Ten day incubation at 100 mg l^{-1} of 4-chlorobiphenyl substrate; data shown are means of duplicate experiments.

[b]Ten day incubation at 10 mg l^{-1} of 4,4'-dichlorobiphenyl substrate; data shown are means of duplicate experiments.

[c]4-Chlorobenzoic acid.

[d]Autoclaved LPS9 inoculum.